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(54) Title: SCREENING METHOD

(57) Abstract: The present invention provides a screening method for the identification of compounds which modulate an AMP-sensitive regulatory site on mitochondria comprising the steps of: a) contacting a test compound with mitochondria in the presence of a substrate for respiration in the presence of a buffer system; b) measuring an index of metabolic rate; and c) identifying compounds which modulate metabolic rate. Alternatively or additionally membrane potential can be measured. The invention also comprises a regulatory site for a mitochondrial proton leak which is related to the adenine nucleotide carrier, a binding assay and a functional assay.

Screening Method

The present invention relates to a novel regulatory site related to the adenine nucleotide carrier that modulates the activity of the mitochondrial proton leak, and to the use of this site in novel screening methods for compounds which are useful in the treatment of body weight disorders, for example obesity and cachexia, and related co-morbid conditions, including, but not limited to, diabetes and dyslipidaemias.

Oxidative phosphorylation is the synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate (P_i) by mitochondria driven by an electrochemical proton gradient that is established during the flow of electrons through the respiratory chain. During electron transfer, protons are pumped from the mitochondrial matrix into the intermembrane space. Protons diffuse back into the matrix through the ATP synthase producing ATP. However, not all of the proton back-flow occurs through ATP synthase. Proton back-flow which is not coupled to ATP synthesis is defined as uncoupling activity. When oligomycin (an inhibitor of proton flow through the membrane domain of ATP synthase) is added to isolated mitochondria, the mitochondria still continue to consume oxygen at a low rate. This process is known as the "proton leak" (Brand et al, *Biochimica et Biophysica Acta* 1187 (1994) 132-139). This proton leak across the mitochondrial inner membrane is estimated to account for as much as 35-50% of skeletal muscle respiration (Rolfe D.F.S. and Brand M.D. (1996) *Am. J. Physiol.* 271S C1380-C1389). In the absence of oxidative phosphorylation all the protons pumped by the respiratory chain out of the mitochondria return into the mitochondria by this proton leak.

The mitochondrial proton leak constitutes a significant proportion of the basal metabolic rate of an organism. Hitherto, there have been no known acutely acting regulators of this biological process. However, an increase of 15% in skeletal muscle mitochondria respiration rate (state 4) caused by supraphysiological concentrations (4.16 mM) of cytidine monophosphate (CMP) has recently been reported (Jekabsons M. and Horwitz B.A. (1998) *FASEB J.* 12, No 5, part II, 4714). The authors suggested a possible CMP regulation of proton leak in skeletal muscle mitochondria. However, our results did not confirm this effect at physiological

concentrations of up to 1mM. In a systematic examination of the effects of fifteen nucleotides on skeletal muscle mitochondria it has surprisingly been found that only one nucleotide, namely adenosine monophosphate (AMP), had an effect at physiologically relevant concentrations. AMP was found to increase the resting
5 respiration rate and double the proton conductance of rat skeletal muscle mitochondria.

The present invention provides screening methods for identifying compounds that modulate the mitochondrial proton leak by interacting at this novel regulatory
10 site. Compounds shown to modulate the proton leak can now be tested in the presence or absence of AMP. A lack of an additive effect indicates that the compound is interacting at this novel site. Compounds which activate this novel AMP regulated proton leak will increase the basal metabolic rate and hence may be useful in treating obesity and related co-morbid conditions. Compounds which inhibit
15 this novel AMP regulated proton leak will decrease the basal metabolic rate and hence may be useful in treating cachexia and related weight loss conditions. In an additional aspect the present invention provides a novel regulatory site for a mitochondrial proton leak wherein the site is activated by adenosine monophosphate (AMP).

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The present invention provides a screening method for the identification of compounds which modulate an AMP-sensitive regulatory site on mitochondria comprising the steps of:

- a) contacting a test compound with mitochondria in the presence of a substrate
25 for respiration in the presence of a buffer system ;
- b) measuring an index of metabolic rate ; and
- c) identifying compounds which modulate the metabolic rate.

Preferably the method further comprises the steps of a) contacting the
30 compounds identified with mitochondria in the presence of AMP and measuring an index of metabolic rate and b) comparing the index of metabolic rate in the presence of AMP and the absence of AMP and thus identifying compounds where there is not an additive effect on metabolic rate as compounds which modulate the AMP-sensitive regulatory site.

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The method may be used for identifying compounds which are suitable for use in the treatment of a body weight disorder. Typically, the method is used for identifying compounds which activate the AMP-sensitive regulatory site on mitochondria in which case in step c) compounds which increase the metabolic rate
5 are identified. Such compounds may be useful in treating obesity and related disorders.

Alternatively, the method is used for identifying compounds which inhibit the AMP-sensitive regulatory site in which case in step c) compounds which decrease
10 the metabolic rate are identified. Such compounds may be useful in treating cachexia and related disorders.

The present invention also provides a screening method for the identification of compounds which modulate an AMP-sensitive regulatory site on mitochondria comprising the steps of:
15

- a) contacting a test compound with mitochondria in the presence of a substrate for respiration in the presence of a buffer system;
- b) measuring the membrane potential; and
- c) identifying compounds which change the membrane potential.

20 Preferably the method which further comprises the steps of a) contacting the compounds identified with isolated mitochondria in the presence of AMP and measuring membrane potential, and b) comparing the membrane potential in the presence of AMP and the absence of AMP and thus identifying compounds where
25 there is not an additive effect on membrane potential as compounds which modulate the AMP-sensitive regulatory site.

Typically, the method is used for identifying compounds which activate an AMP-sensitive regulatory site on mitochondria in which case in step c) compounds
30 which decrease the membrane potential are identified. Such compounds may be useful in treating obesity and related conditions.

Alternatively, the method is used for identifying compounds which inhibit the AMP-sensitive regulatory site in which case in step c) compounds which increase the
35 membrane potential are identified. Such compounds may be useful in treating cachexia and related conditions.

The present invention also provides a screening method for the identification of compounds which modulate an AMP-sensitive regulatory site on mitochondria comprising the steps of:

- 5 a) contacting a test compound with mitochondria in the presence of a substrate for respiration in the presence of a buffer system;
- b) measuring the metabolic rate and measuring the membrane potential; and
- c) identifying compounds which change the metabolic rate and which change the membrane potential.

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Preferably the method further comprises the steps of a) contacting the compounds identified with mitochondria in the presence of AMP and measuring the metabolic rate and measuring the membrane potential; and b) comparing the metabolic rate and the membrane potential in the presence of AMP and the absence

15 of AMP and thus identifying compounds where there is not an additive effect on metabolic rate and the membrane potential as compounds which activate the AMP-sensitive regulatory site.

Typically, the method is used for identifying compounds which activate an

20 AMP-sensitive regulatory site on mitochondria in which case in step c) compounds which increase the metabolic rate and which decrease the membrane potential are identified. Such compounds may be useful in treating obesity and related conditions.

In one embodiment the present invention provides a screening method for

25 the identification of compounds which activate an AMP-sensitive regulatory site on mitochondria comprising the steps of:

- a) contacting a test compound with mitochondria in the presence of a substrate for respiration in the presence of a buffer system ;
- b) measuring the oxygen consumption; and
- 30 c) identifying compounds which increase oxygen consumption.

Preferably the method further comprises the steps of a) contacting the compounds identified with mitochondria in the presence of AMP and measuring the oxygen consumption; and b) comparing the oxygen consumption in the presence of

35 AMP and the absence of AMP and thus identifying compounds where there is not an

additive effect on oxygen consumption as compounds which activate the AMP-sensitive regulatory site.

The present invention also provides a screening method for the identification of compounds which activate an AMP-sensitive regulatory site on mitochondria comprising the steps of:

- a) contacting a test compound with mitochondria in the presence of a substrate for respiration in the presence of a buffer system;
- b) measuring the membrane potential; and
- 10 c) identifying compounds which decrease the membrane potential.

Preferably the method which further comprises the steps of a) contacting the compounds identified with isolated mitochondria in the presence of AMP and measuring membrane potential, and b) comparing the membrane potential in the presence of AMP and the absence of AMP and thus identifying compounds where there is not an additive effect on membrane potential as compounds which activate the AMP-sensitive regulatory site.

The present invention also provides a screening method for the identification of compounds which activate an AMP-sensitive regulatory site on mitochondria comprising the steps of:

- a) contacting a test compound with mitochondria in the presence of a substrate for respiration in the presence of a buffer system;
- b) measuring the oxygen consumption and measuring the membrane potential; and
- 25 c) identifying compounds which increase oxygen consumption and which decrease the membrane potential.

Preferably the method further comprises the steps of a) contacting the compounds identified with mitochondria in the presence of AMP and measuring the oxygen consumption and measuring the membrane potential; and b) comparing the oxygen consumption and the membrane potential in the presence of AMP and the absence of AMP and thus identifying compounds where there is not an additive effect on oxygen consumption and the membrane potential as compounds which activate the AMP-sensitive regulatory site.

Alternatively, the method is used for identifying compounds which inhibit an AMP-sensitive regulatory site on mitochondria in which case in step c) compounds which decrease the metabolic rate and which increase the membrane potential are identified. Such compounds may be useful in treating cachexia and related conditions.

Suitably, the mitochondria are isolated mitochondria or are present in intact cells. Preferably, the mitochondria are isolated skeletal muscle mitochondria. More preferably the mitochondria are isolated rat skeletal muscle mitochondria. Preferably the mitochondria are present in intact eukaryotic cells or are present in tissue slices of mammalian origin or cell lines of mammalian origin.

Unless the context indicates otherwise when the term "mitochondria" is used in connection with a screening method of the invention, the term also includes any suitable part or derivative of mitochondria.

By "suitable part or derivative of mitochondria" we mean any part or derivative thereof which contains the AMP-sensitive regulatory site and which undergoes metabolism which can be measured as described herein and which has a membrane potential which can be measured. A suitable part or derivative of mitochondria includes submitochondrial vesicles and can also include reconstitution of purified components (such as the ANC) into artificial membrane systems. There are a variety of methods well known in the art for both preparing these vesicles and for assaying transport activity thereafter (for example Methods in Enzymology Volume LV 1979: Bioenergetics: Oxidative Phosphorylation ed. S Fleisher & L Packer). In particular, membrane vesicles include vesicles of the inner mitochondrial membrane.

Unless the context indicates otherwise when the term "membrane potential" is used in connection with a screening method of the invention, the term also includes the pH gradient which is the other component of proton motive force. Thus, in the relevant screening methods the pH gradient may be measured instead of or as well as the membrane potential. It is preferred if the membrane potential is measured.

Suitably any substrate for respiration may be used. Preferably the substrate for respiration is a succinate salt, a glutamate salt or a malate salt, for example as a potassium salt or a sodium salt. More preferably, the substrate is a succinate salt,

for example potassium succinate or sodium succinate. Preferably the screening method is carried out in the presence of an inhibitor of the utilisation of other endogenous substrates (a complex 1 inhibitor). When a succinate salt is employed then the screening method is preferably carried out in the presence of the complex 1
5 inhibitor rotenone to inhibit the utilisation of other endogenous substrates.

Preferably, the screening method is carried out in varying concentrations of an electron transport inhibitor. More preferably, the electron transport inhibitor is selected from a malonate salt, myxothiazol or a cyanide salt. Most preferably, the
10 electron transport inhibitor is a malonate salt, for example the sodium or the potassium salt.

Suitably any index of metabolic rate known to those skilled in the art may be used. Preferably the index of metabolic rate is selected from the following: cell
15 growth rate, oxygen consumption; heat production, free radical production, lactate production, glucose utilisation or carbon dioxide emission [Experimental procedures for measuring these indices of metabolic rate are found in "Obesity" editors P. Bjorntorp and B.N. Brodoff published by J.B. Lippincott Company 1992 Chapter 8 "The Biochemistry of Energy Expenditure" by J.P. Flatt, and references cited
20 therein]. More preferably the index of metabolic rate is oxygen consumption.

When membrane potential is being measured then the screening method is preferably carried out in the presence of a proton/potassium exchanger, for example nigericin, to minimise the pH gradient.
25

Membrane potential is preferably measured using a) ion selective electrodes for methyltriphenylphosphonium cation (TPMP) (or tetraphenylphosphonium cation (TPP)) wherein TPMP (or TPP) has been added to the test system b) by using fluorescent membrane potential dyes wherein changes in membrane potential are
30 measured by a fluorimeter which records the changes in the fluorescent response due to partitioning or c) radiolabelled TPMP or TPP. A suitable dye is (dimethyl(aminostyryl)-1-methyl pyridinium (DSMP)) which may be used to measure membrane potential in both intact cells and isolated mitochondria. The pH gradient can be measured using methods that are well known in the art for example a) pH
35 electrodes b) fluorescent dyes or c) radiolabelled probes.

Preferably the oxygen consumption and/or the membrane potential measurements are carried out in the presence of an inhibitor of ATP synthesis for example oligomycin.

- 5 Preferably the oxygen consumption is measured using an oxygen electrode.

10 The term buffer system is used herein to mean a system capable of supporting mitochondria and comprises a buffering agent, for example HEPES, and an osmotic protector, for example KCl. The buffering system optionally further comprises a chelating agent, for example EGTA, and/or an inorganic phosphate, for example potassium dihydrogen phosphate, and/or a free fatty acid scavenger, for example defatted BSA.

15 The aforementioned screening methods of the invention may be used for the identification of compounds which are suitable for use in the treatment of a body weight disorder. In particular, the methods which identify compounds which activate an AMP-sensitive regulatory site on mitochondria may be used for the identification of compounds which are suitable for use in the treatment of obesity and related disorders. The methods which identify compounds which inhibit an AMP-sensitive
20 regulatory site on mitochondria may be used for the identification of compounds which are suitable for use in the treatment of cachexia and related disorders.

25 Preferably, in the methods of the invention, compounds with an undesirable activity profile and which decrease membrane potential and decrease metabolic rate (eg oxygen consumption) are likely to be metabolic poisons and are discarded. Also, stimulators of respiration would increase membrane potential and increase metabolic rate (eg oxygen consumption) are also discarded.

30 Compounds may not be useful if they activate the mitochondrial permeability transition pore or inhibit the ADP/ATP exchange activity of the ANC. The effect the compounds on the ADP/ATP exchange activity of the ANC can be assayed using methods described in M Stubbs 'Inhibitors of the adenine nucleotide translocase' 1979 Pharmac. Ther. 7:329-349.

Further work has shown that this AMP effect is antagonised by inhibitors and substrates of the adenine nucleotide carrier (ANC) suggesting that the ANC is the molecular mediator of this AMP effect.

5 The mitochondrial adenine nucleotide carrier (ANC) is a key link in the oxidative function of organisms in that it exchanges ATP generated inside the mitochondria for ADP in the cytosol (reviewed C Fiore et al, 1998 *Biochimie* 80: 137-150). Inhibition by certain agents (such as atractylate and bongkrekate) block this function, prevent aerobic metabolism and at higher doses cause death resulting from
10 the inability of mitochondria to generate ATP. Genetic analysis indicates that there are 3, highly conserved, ANC isoforms encoded by 3 different genes in mammals termed ANC1, 2 and 3 respectively. In mammalian species it appears that ANC2 is the key isoform as it is ubiquitously expressed in all tissues and expression is induced in variable amounts depending on the respiratory activity of the tissue.
15 ANC1, in contrast, is expressed in very high amounts predominantly in heart and skeletal muscle and genetic knock-out data (BH Graham et al. *Nature Genetics* 1997:16: 226-234) indicates that this isoform is important for aerobic metabolism in these tissues only. ANC3, on the other hand, is expressed at only very low levels in some tissues (if at all) and would appear to have little functional relevance. Lower
20 organisms (including insects, yeast and plants) also have a requirement for an ANC and have 3 isoforms though of lower homology. The ANC is a member of the well characterised mitochondrial transporter family that all have molecular weights of approximately 32,000 Da.

25 It has become clear recently that, at least in vitro, the ANC has other activities, involving ion flux, apart from the ADP/ATP exchange reaction. Evidence using reconstituted purified ANC now suggests that this protein is also a key component of the mitochondrial permeability transition pore that is activated in apoptosis (N Brustovetsky & M Klingenberg 1996 *Biochemistry* 35: 8483-8488). This
30 permeability pore opening is activated by atractylate and inhibited by bongkrekic acid (both ADP/ATP exchange inhibitors). Evidence also suggests that this protein is involved in, at least part of, the observed uncoupling effects of free fatty acids by acting as a pore for the ionised acid (reviewed V Skulachev 1998 *Biochim. Biophys. Acta* 1363: 100-124). These data suggest that, at least under some circumstances,
35 the ANC shares some activity with the related uncoupling proteins. There is no teaching or suggestion in the prior art that the ANC is involved in obesity.

To date, the ANC has not represented a potential pharmacological target for stimulating metabolic rate as the permeability pore or the fatty acid mediated uncoupling is not amenable to pharmacological manipulation (toxicity and non-specific activity respectively). This is the first demonstration that specific activation of the uncoupling activity of the ANC is possible. As the activation of this uncoupling activity manifests itself as a substantial increase in proton leak this mechanism can be used to increase basal metabolic rate for the treatment of obesity and diabetes and related conditions.

10

In another aspect the present invention provides a novel regulatory site on the adenine nucleotide carrier which is regulated by AMP and which activates a novel proton leak. In a preferred aspect this novel regulatory site is present on ANC1. In addition the present invention also provides the use of this novel regulatory site in the identification of compounds which are useful in the treatment of body weight disorders such as obesity and related conditions and cachexia.

In a further aspect the present invention provides a screening method for the identification of compounds which are useful in the treatment of a body weight disorder the method comprising the step of identifying a compound which selectively binds to ANC. In one embodiment, the compound decreases the proton leak activity of ANC. For example, the compound may bind substantially reversibly or substantially irreversibly to the active site of ANC. In a further embodiment, the compound may bind a portion of ANC that is not the active site so as to interfere with the binding of ANC to its substrate. In a still further embodiment, the compound may bind a portion of ANC so as to decrease the activity of ANC by an allosteric effect.

The invention provides a screening method for the identification of compounds which are useful in the treatment of obesity and related conditions the method comprising the step of identifying an agonist of the AMP effect on the ANC mediated proton leak.

The invention also provides a screening method for the identification of compounds which are useful in the treatment of cachexia and related conditions the method comprising the step of identifying an antagonist of the AMP effect on the ANC mediated proton leak.

In a further aspect the present invention provides a binding assay for the identification of compounds which are suitable for use in the treatment of obesity and related conditions comprising the steps of:

- 5 a) incubating an ANC-containing preparation with a labelled ligand to produce a labelled ANC-containing preparation;
- b) contacting a test compound with the labelled ANC-containing preparation; and
- 10 c) identifying a compound which reduces the amount of labelled ligand present in the ANC-containing preparation as a compound which may suitable for use in the treatment of obesity.

In a further aspect, the invention provides a screening method for the identification of compounds which are useful in the treatment of a body weight disorder the method comprising the step of identifying a compound which modulates the mitochondrial proton leak mediated by an ANC.

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In yet another aspect the present method also provides a functional screening method for identifying compounds which modulate the mitochondrial proton leak mediated by an ANC comprising the steps of:

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- a) incubating a test compound with cells containing an ANC and measuring an index of metabolic rate and/or membrane potential
- 25 b) incubating a test compound with control cells in which the ANC used in step a) is absent or is present at lower levels than in step a) and measuring an index of metabolic rate and/or membrane potential and
- 30 c) identifying a compound which gives rise to a different metabolic rate and/or different membrane potential in step a) compared to step b) as a compound which modulates the mitochondrial proton leak mediated by an ANC.

Preferably the control cells show no AMP effect.

Typically, a compound which gives rise to an increased metabolic rate and/or decreased membrane potential in step c) is identified as a compound which enhances the proton leak mediated by ANC.

- 5 Alternatively, a compound which gives rise to a decreased metabolic rate or increased membrane potential is identified as a compound which reduces proton leak mediated by an ANC.

10 Compounds identified as modulating the proton leak mediated by an ANC may be useful in treating body weight disorders. Compounds identified as enhancing the proton leak may be useful in treating obesity and related disorders whereas compounds identified as reducing the proton leak may be useful in treating cachexia and related disorders.

15 Suitably the ANC-containing preparation comprises one of the following: a) intact tissue preparations for example mammalian tissue preparations eg human , bovine or rodent skeletal muscle or cardiac tissue; b) cell lines, from a skeletal muscle source eg mouse C2C12, G7, G8 or L7 cells, rat L6 or L8 cells, or human SJCRH30; or from a cardiac source eg rat H9c2 (2-1) cells, or from an aortic smooth
20 muscle source eg rat A7r5, or human T/G HA-VSMA; c) cells (for example yeast cells) into which ANC has been introduced by genetic means d) cells isolated from tissues for example from cardiac or skeletal muscle tissues e) membranes ; f) mitochondria ; g) mitochondrial membranes or h) isolated ANC preferably in purified form.

25

 Preferably the ANC-containing preparation is prepared using gene cloning techniques. Using transfection techniques the nucleotide sequence encoding an ANC polypeptide which modulates the ANC mediated mitochondrial proton leak may be introduced into a cellular host for example a mammalian cell line or yeast cells to
30 increase the level of ANC activity (upregulation) or to introduce an ANC isoform which was not present initially, using techniques known to those skilled in the art. An ANC-containing preparation may then be isolated from these cells. Alternatively other means of increasing the amount of ANC may be employed. Animals may be subjected to pharmacological treatment, for example with thyroid hormones e.g. T₃
35 or T₄ , or environmental stress for example heat or cold or over-feeding prior to removal of the ANC-containing preparation. The ANC-containing preparation may

be collected from genetically modified strains of animals. eg ob/ob mice, Zucker rats and strains in which certain genes and proteins have been inactivated by generic means including deletions of mitochondrial genes. Alternatively cells may be treated with altered growth serum or with pharmacological agents, for example with thyroid hormones, e.g. T₃ or T₄.

Preferably the ANC-containing preparation is a cell line or a membrane preparation from a cell line or from tissues. More preferably the ANC-containing preparation is a cell line which is optionally upregulated.

Suitably the cells containing an ANC include but are not limited to a) cell lines, from a skeletal muscle source eg mouse C2C12, G7, G8 or L7 cells, rat L6 or L8 cells, or human SJCRH30; or from a cardiac source eg rat H9c2 (2-1) cells, or from an aortic smooth muscle source eg rat A7r5, or human T/G HA-VSMA; b) cells (for example yeast cells) into which ANC has been introduced by genetic means c) cells isolated from tissues for example from cardiac or skeletal muscle tissues or d) isolated mitochondria from a) b) or c) immediately above. The cells containing an ANC are preferably upregulated in a similar manner to that described above for the ANC-containing preparation. It will be understood by those skilled in the art that the control cells are preferably from the same source as the test cells.

Suitably the ANC-containing preparation comprises ANC1, ANC2 or ANC3 or mixtures thereof. Preferably the ANC-containing preparation comprises ANC1.

Suitably the labelled ligand is a compound which interacts with the ANC which has been radiolabelled or fluorescently labelled by methods known to those skilled in the art or a compound which is an inhibitor of ANC as a result of fluorescent labelling. Suitable inhibitors of the ATP/ADP exchange activity of the ANC which may be radiolabelled or fluorescently labelled include but are not limited to the following: atractylate, carboxyatractylate and bongkrekate. Suitable compounds which are inhibitors of the ATP/ADP exchange activity of the ANC as a result of fluorescent labelling include but are not limited to the following: fluorescently labelled ATP, ADP or AMP. Suitable substrates for ANC which may be radiolabelled or fluorescently labelled include but are not limited to the following: ADP or ATP. Preferably the labelled ligand is radiolabelled or fluorescently labelled atractylate or fluorescently labelled ATP or ADP. More preferably the labelled ligand is

radiolabelled or fluorescently labelled atractylate. Most preferably the labelled ligand is radiolabelled atractylate.

5 It will be understood by those skilled in the art that where the ligand is radiolabelled that an additional filtration or centrifugation step and /or washing step is required to remove unbound label. It will be understood by those skilled in the art that prior treatment of the cells used in the binding assay may be required to facilitate permeability of the cells to the labelled ligand for example pre-treatment with detergent.

10

This screening method has the advantage that it may be used in high-throughput screening assays to test large numbers of compounds quickly and hence identify compounds which may be suitable for use in the treatment of obesity and related conditions.

15

The term "associated co-morbid conditions" as used in this document means medical conditions known to those skilled in the art to be associated with body weight disorders. The term includes but is not limited to the following: diabetes including non-insulin dependent diabetes mellitus, impaired glucose tolerance, lipid syndromes, cachexia, hyperglycaemia and hyperlipidaemia, high uric acid levels and lipid levels, in mammals particularly humans.

20

In addition the present invention may be useful in identifying compounds for the treatment or prevention of metabolic diseases and conditions arising therefrom, for example increased non exercise activity thermogenesis and increased metabolic rate, weight gain associated with drug treatment, osteoarthritis and gout, cancers associated with weight gain, menstrual dysfunction or gallstones.

25

The present invention may be useful in identifying compounds for preventing cardiovascular disease, in aiding weight loss after pregnancy and in aiding weight loss after smoking cessation.

30

Cachexia is used to denote a state of constitutional disorder, malnutrition and general ill-health. The chief signs of this condition are bodily emaciation, sallow unhealthy skin and heavy lustreless eyes. Specific examples include but are not limited to cancer and AIDs induced cachexia.

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It will be understood by those skilled in the art that the isolated ANC used may be either purified protein or recombinant protein obtained by methods known to those skilled in the art and as described below. In addition purified ANC may be reconstituted in artificial membrane structures (eg liposomes) to further characterize its function and its regulation.

Improved assay methods for ANC can be developed. For example, the three dimensional structure of active sites on the protein may be identified by crystallography which will increase knowledge of the structure and function. Computer modelling may be used to identify compounds which are likely to interact at these active sites and hence increase the chances of identifying compounds which have useful therapeutic properties. A specific assay could be developed to identify compounds which interact at these active sites.

It is preferred if a compound selected by any of the aforementioned screening methods or assays of the invention as being suitable for the treatment of obesity or a related condition is screened in a further screen for suitability in treating obesity or a related condition. In particular, it is preferred if the compound is screened in an animal model of obesity and that compounds which have a desired effect in this model (eg are shown to reduce or prevent obesity to a useful extent) are selected for further study or use in treatment.

In particular, it is preferred if the compound is screened in an animal model used for measuring basal metabolic rate (I. Connoley et al 1999 Br J Pharmacol 126:1487-1495) and that compounds which have a desired effect in this model (increased for obesity and decreased for cachexia) are selected for further study.

Similarly, it is preferred if a compound selected by any of the aforementioned screening methods and assays of the invention as being suitable for the treatment of cachexia is screened in a further screen for suitability in treating cachexia. In particular, it is preferred if the compound is screened in an animal model of cachexia and that compounds which have a desired effect in this model (eg are shown to reduce or prevent cachexia to a useful extent) are selected for further study or use in treatment.

It will be appreciated that in the methods described herein, which may be drug screening methods, a term well known to those skilled in the art, the compound selected may be a drug-like compound or lead compound for the development of a drug-like compound.

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The term "drug-like compound" is well known to those skilled in the art, and may include the meaning of a compound that has characteristics that may make it suitable for use in medicine, for example as the active ingredient in a medicament. Thus, for example, a drug-like compound may be a molecule that may be synthesised by the techniques of organic chemistry, less preferably by techniques of molecular biology or biochemistry, and is preferably a small molecule, which may be of less than 5000 daltons and which may be water-soluble. A drug-like compound may additionally exhibit features of selective interaction with a particular protein or proteins and be bioavailable and/or able to penetrate target cellular membranes, but it will be appreciated that these features are not essential.

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The term "lead compound" is similarly well known to those skilled in the art, and may include the meaning that the compound, whilst not itself suitable for use as a drug (for example because it is only weakly potent against its intended target, non-selective in its action, unstable, poorly soluble, difficult to synthesise or has poor bioavailability) may provide a starting-point for the design of other compounds that may have more desirable characteristics. It will be appreciated that the screening methods and assays of the invention may identify lead compounds.

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A further aspect of the invention provides a compound identifiable or identified in a screening method or assay of the invention. A still further aspect provides the compound for use in medicine, that is to say packaged and presented for use in medicine.

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The compound may be useful in treating a body weight disorder and so a further aspect of the invention provides a method of treating a body weight disorder in a patient the method comprising administering to the patient a compound identifiable or identified in the screening methods or assays of the invention.

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The invention also provides use of a compound identifiable or identified in the screening methods or assays of the invention in the manufacture of a medicament for treating a body weight disorder.

5 Compounds which are identified or identifiable in screens or assays which are useful for finding compounds for treating obesity (as detailed above) are used in the method of treatment for treating obesity or related conditions. Compounds which are identified or identifiable in screens or assays which are useful for finding
10 compounds for treating cachexia (as detailed above) are used in the method of treatment for treating cachexia or related conditions.

 A further aspect of the invention provides a method of treating a patient with obesity or a related co-morbid condition the method comprising administering to the patient an agonist of an AMP-sensitive regulatory site on mitochondria or an agonist
15 of the AMP effect on the ANC mediated proton leak.

 A still further aspect of the invention provides use of an agonist of an AMP-sensitive regulatory site on mitochondria or an agonist of the AMP effect on the ANC mediated proton leak in the manufacture of a medicament for treating obesity or a
20 related co-morbid condition.

 It has been found that three AMP analogues namely 6-chloropurineriboside 5'-monophosphate, cordecypin 5'-monophosphate and xanthosine 5'-monophosphate demonstrated significant activity in decreasing membrane potential
25 and therefore have potential use in the treatment of obesity and related co-morbid conditions

 In another aspect the present invention provides a method of treating a patient with obesity or a related co-morbid condition the method comprising
30 administering to the patient an agonist of an AMP-sensitive regulatory site on mitochondria or an agonist of the AMP effect on the ANC mediated proton leak.

 In yet another aspect the present invention provides the use of an agonist of an AMP-sensitive regulatory site on mitochondria or an agonist of the AMP effect on
35 the ANC mediated proton leak in the manufacture of a medicament for treating obesity or a related co-morbid condition.

In another aspect the present invention provides a protein which is involved in the AMP activated proton leak. In yet another aspect the present invention provides methods for identifying this protein or proteins. The term "involved in" covers proteins on which AMP has a direct action and which directly affects the proton leak and proteins which interact with AMP and then affect the proton leak by indirect means.

A first method of identifying a protein which is involved in the AMP activated proton leak comprises the steps of:

- a) contacting AMP or an analogue thereof with mitochondria in the presence of a substrate for respiration in the presence of a buffer system;
- b) measuring the oxygen consumption and/or the membrane potential;
- c) contacting AMP or an analogue thereof with mitochondria in the presence of a substrate for respiration in the presence of a buffer system in the presence of a known specific inhibitor of a mitochondrial protein;
- d) measuring the oxygen consumption and/or the membrane potential;
- e) identifying a protein whose inhibitor reduces the increase in oxygen consumption and/or the decrease in membrane potential caused by AMP as a protein which is involved in the AMP activated proton leak.

The term "an analogue thereof" means a compound which has a similar effect on the proton leak as AMP or has higher affinity.

It will be understood by those skilled in the art that the necessary control experiments are required to validate this method. For example it would be desirable to incubate the mitochondria with the specific protein inhibitor to ensure that there was no direct effect of the protein inhibitor on the proton leak. It would also be desirable to incubate the mitochondria in the buffer system as a control.

A second method of identifying a protein which is involved in the AMP activated proton leak comprises the steps of:

- a) contacting AMP or an analogue thereof with mitochondria of modified protein composition_ in the presence of a substrate for respiration in the presence of a buffer system;

- b) comparing the effect of AMP or a high affinity analogue on oxygen consumption and/or the membrane potential on mitochondria with modified protein composition with control mitochondria;
- 5 c) identifying modifications in protein composition which affect the increase in oxygen consumption and/or the decrease in membrane potential caused by AMP and hence identifying the corresponding protein/s which are involved in the AMP activated proton leak.

10 The mitochondria of modified protein composition may be obtained by methods known to those skilled in the art, for example, the mitochondria may be collected from animals which have been subjected to a stress eg overfeeding , underfeeding, heat, cold , restricted movement etc. or whose environment has been otherwise manipulated. Alternatively dosing of animals with pharmacological agents may be used to alter mitochondrial protein composition. Alternatively the
15 mitochondria may be collected from genetically modified strains of animals.eg ob/ob mice, Zucker rats and strains in which certain genes and proteins have been inactivated by generic means including deletions of mitochondrial genes.

20 A third method of identifying a protein which is involved in the AMP activated proton leak comprises the steps of:

- a) extracting the proteins from mitochondria and separating and purifying these proteins by methods known to those skilled in the art;
- b) incubating the separated proteins with a fluorescent, radiolabelled or other labelled AMP or analogue in a suitable binding assay using methods known
25 to those skilled in the art;
- c) identifying those pure proteins which bind labelled AMP or analogue as being proteins involved in the AMP activated proton leak and
- d) partially sequence the purified protein and using techniques known to those skilled in the art construct probes to allow the gene to be identified.

30

A fourth method of identifying a protein which is involved in the AMP activated proton leak comprises the steps of:

- a) extracting the proteins from mitochondria and incubating them with a photoaffinity labelled version of AMP or analogue using methods known to
35 those skilled in the art to label proteins;

- b) thereafter extracting, isolating and purifying those labelled proteins by methods known to those skilled in the art
- c) identifying the structure of these proteins; and
- d) identifying these proteins as proteins involved in the AMP activated proton leak; and
- 5 e) partially sequence the purified protein and using techniques known to those skilled in the art construct probes to allow the gene to be identified.

10 A preferred method of purifying a protein which is involved in the AMP activated proton leak comprises passing extracted proteins, obtained as described previously, through a column containing immobilised AMP or an analogue thereof.

15 In a further aspect the present invention provides a screening method for the identification of compounds which activate an AMP-sensitive regulatory site on mitochondria comprising the steps of:

- a) contacting a test compound with a protein which is involved in the AMP activated proton leak in a protein binding assay and identifying compounds with a high binding affinity as compounds which activate an AMP-sensitive regulatory site on mitochondria.

20

It will be understood by those skilled in the art that the protein used may be either purified protein or recombinant protein obtained by methods known to those skilled in the art and as described below.

25 Once the protein has been identified improved assay methods can be developed. For example, the three dimensional structure of active sites on the protein may be identified by crystallography which will increase knowledge of the structure and function. Computer modelling may be used to identify compounds which are likely to interact at these active sites and hence increase the chances of
30 identifying compounds which have useful therapeutic properties. A specific assay could be developed to identify compounds which interact at these active sites.

In addition the target protein may be reconstituted in artificial membrane structures (eg liposomes) to further characterize function and its regulation.

35

In another aspect the present invention provides a gene which expresses a protein which is involved in the AMP activated proton leak. In yet another aspect the present invention provides a method for identifying this gene.

- 5 A method for identifying a gene which expresses a protein which is involved in the AMP activated proton leak comprising the steps of :
- a) searching genomic databases and identifying genes which have an AMP binding domain;
 - b) expressing these genes in cells;
 - 10 c) isolating the mitochondria from said cells; and
 - d) determining which mitochondria have an altered response to AMP (when compared to control) and hence determine which of these genes are responsible for the AMP activated proton leak.

- 15 Once the gene has been identified improved assay methods may be developed as follows. Firstly an assay method may be utilised in which, once a promoter region of the gene has been identified by methods known to those skilled in the art, the gene may be upregulated to produce additional protein in a target cell thus increasing the sensitivity of the assay. In other words the protein may be
- 20 overexpressed in a suitable expression system as known to those skilled in the art. Secondly the AMP regulated proton leak may be generated in cells which do not normally have it and such cells may be used in the screening assay.

- In an additional aspect the present invention provides a regulatory site for a
- 25 mitochondrial proton leak wherein the site is activated by adenosine monophosphate (AMP).

- In another aspect the present invention provides the gene identified by the above methods.

- 30 In a further aspect the present invention provides the protein which acts as a regulatory site for a mitochondrial proton leak wherein the site is activated by AMP.

- In a yet further aspect the present invention provides the protein identified by
- 35 the methods identified above.

EXPERIMENTAL PROCEDURES

The effect of adenosine, guanosine, cytidine, thymidine and uridine mono- di- and triphosphates at a concentration of 1 mM on the state 4 respiration rate (defined below) and proton leak of rat skeletal muscle mitochondria was studied.

Isolation of skeletal muscle mitochondria.

Female Wistar rats (4-8 wk old) were killed by stunning followed by cervical dislocation and the skeletal muscle was immediately dissected from the hindlimbs, weighed and placed in pre-weighed beaker containing C-P 1 medium (0.1 M KCl, 0.05 M Tris-HCl, 2 mM EGTA, pH 7.4). Mitochondria were isolated according to the methods of Chappell J.B. and Perry S.V. (1954) *Nature* (London) 173, 1094-1095 and Bhattacharya *et al.* (1991) *Anal. Biochem.* 192, 344-349. Briefly, the tissue was placed on a pre-cooled porcelain tile and shredded with a sharp blade. The tissue was minced and washed further by chopping with a sharp scissors and rinsing with C-P 1 medium 4-5 times and then drained of medium and left stirring in a beaker on ice containing C-P 2 medium (0.1 M KCl, 0.05 M Tris-HCl, 2 mM EGTA, 1 mM ATP, 5 mM MgCl₂, 0.5% bovine serum albumin (BSA), and 18.7 U protease (nagarse)/g tissue, pH 7.4) for 4 min. The tissue was homogenised in the same medium using a Polytron tissue homogeniser. The homogenised tissue was left stirring in the same medium on ice for a further 6 min and then was centrifuged at 490g for 10 min. The supernatant was filtered through muslin and again centrifuged at 10368g for 10 min. The mitochondrial pellets were resuspended in C-P 1, combined and centrifuged again at 10368g for 10 min. A last centrifugation was performed at 3841g and finally the pellet was resuspended in approx. 500 µl of C-P 1. Protein concentration was determined by the Biuret method.

Measurement of Oxygen Consumption.

The respiration rate was measured in the absence of adenosine diphosphate (ADP) (state 4) and presence of oligomycin (to inhibit any ATP synthesis) as a crude indicator of mitochondrial proton conductance. Oxygen consumption was measured using a Clark-type oxygen electrode (Hansatech, Great Britain) maintained at 37°C. Prior to any experimental runs, the linearity of the oxygen electrode was routinely checked by measuring the uncoupled rate (i.e. the respiratory rate in the presence of

the uncoupler (FCCP) at 0.2 μ M) and the oxygen electrode was calibrated with the appropriate volume of oxygenated medium (i.e. medium equilibrated with air). The oxygen concentration of air-saturated medium at 37°C was assumed to be 406 nmol/ml (Reynafarje B *et al.* (1985). *Anal. Biochem.* 145, 406-418). Oxygen
5 consumption was measured in the absence (state 4) and in the presence (state 3) of 250 μ M ADP. The skeletal muscle mitochondria respiratory control ratio (state 3/state 4 oxygen consumption) was around 4.0 with succinate as substrate. For the measurements, 0.5 mg of mitochondrial protein per ml of assay medium (120 mM KCl, 5 mM KH_2PO_4 , 3 mM HEPES, 1 mM EGTA and 0.3% defatted BSA, pH 7.2)
10 was added to the oxygen electrode chamber followed by 5 μ M rotenone, 1 μ g/ml oligomycin and 4 mM succinate. Afterwards, adenosine, guanosine, cytidine, thymidine and uridine mono- di- and triphosphates were added to the oxygen electrode chamber at a concentration of 1 mM. The pH of each nucleotide solution was brought to 6-7 so that no modification in the pH of the reaction mixture occurred
15 after their addition.

Measurement of proton leak.

The rate at which protons cycle across the mitochondrial inner membrane,
20 which does not contribute to ATP synthesis by oxidative phosphorylation, is given by the relationship observed between mitochondrial membrane potential and oxygen consumption rate during titration with electron transport chain inhibitors. This is a non-linear relationship, which suggests that the rate of dissipation of redox energy varies with membrane potential (Brown G.C. and Brand M.D. (1991) *Biochim.*
25 *Biophys. Acta* 1059, 55-62). The respiration rate and mitochondrial membrane potential were determined simultaneously using oxygen electrodes and electrodes sensitive to the potential-dependent probe TPMP⁺, and established the kinetic response of the proton leak to potential during titrations of the potential with inhibitors of electron transport (Brand M.D. (1995) *Bioenergetics. A practical approach* (Brown
30 G.C. and Cooper C.E., eds.), IRL Press, pp. 39-62). For each run, 0.5 mg of mitochondrial protein per ml of assay medium (120 mM KCl, 5 mM KH_2PO_4 , 3 mM HEPES, 1 mM EGTA and 0.3% defatted BSA, pH 7.2) was added to the oxygen electrode chamber. Prior to measurements the electrode was calibrated with sequential additions of up to 2 μ M TPMP. 5 μ M rotenone was added to prevent
35 respiration on endogenous NAD-linked substrates. 1 μ g/ml oligomycin inhibited

mitochondrial ATP synthase and 75 ng/ml nigericin was added to bring the difference in pH across the inner mitochondrial membrane close to zero. 4 mM succinate was used as substrate. Additions of malonate up to 2 mM were sequentially performed. At the end of each run, the uncoupler FCCP at 0.2 μ M was added to dissipate the membrane potential, so that the TPMP was released by the mitochondria back to the medium. TPMP binding correction for skeletal muscle was taken to be 0.35 (μ l/mg protein)⁻¹ (Rolfe D.F.S. *et al.* (1994) *Biochim. Biophys. Acta* 1118, 405-416.).

The proton leak activity of a number of AMP analogues was measured using the membrane potential sensitive fluorescent dye, DSMP.

Determination of free Mg²⁺ concentration.

Free Mg²⁺ concentration was determined from total Mg²⁺ in the assay medium. Apparent stability constants were calculated from absolute stability constants taken from (Fabiato A. and Fabiato A. (1979) *J. Physiol. Paris*, 75, 463-505) at a precise temperature and ionic strength.

Results

In the presence of oligomycin (to inhibit the ATP synthase), AMP stimulated the respiration of rat skeletal muscle mitochondria by $55 \pm 1.9\%$ ($n = 15; p < 0.001$). AMP activation showed simple saturation (Figure 1), with the half-maximal effect at 80 μ M AMP (Figure 1b), which is in the physiological range (Arabadjis, P.G., Tullson, P.C. & Terjung, R.L. *Am. J. Physiol.* **264**, C1246-C1251 (1993)). Stimulation by AMP was unaffected by addition of 2 mM Mg, 1 mM EDTA, or 50 μ M diadenosine pentaphosphate (to prevent AMP metabolism through adenylate kinase) or by pH between 6.5 and 7.3. AMP activation was unaffected by removal or addition of albumin, suggesting that it is not dependent on free fatty acids. There was no effect on respiration of ADP, ATP or the nucleoside monophosphates or diphosphates of guanine, cytosine, thymine or uracil at 1 mM. In the absence of added Mg or EDTA the nucleoside triphosphates each stimulated respiration by 60%, but this was entirely explained by chelation of endogenous contaminating Mg, which is a potent inhibitor of the basal proton conductance in muscle mitochondria (Cadenas, S., Jones, R.B. & Brand, M.D. 8th Int. Congr. Obesity, Paris. Abstract HTP10 (1998)).

To test whether AMP directly activates mitochondrial proton conductance, its kinetics were assayed by measuring the response of the proton leak rate to its driving force, mitochondrial membrane potential (Brand, M.D., Chien, L.-F., Ainscow, E.K., Rolfe, D.F.S. & Porter, R.K. *Biochim. Biophys. Acta* **1187**, 132-139 (1994).
5 Brand, M.D., Brindle, K.M., Buckingham, J.A., Harper, J.A., Rolfe, D.F.S. & Stuart, J.A. *Int. J. Obesity* **23**, Suppl. 6, S3-S11 (1999), Brand, M.D. *Mol. Cell. Biochem.* **184**, 13-20 (1998)). 1 mM AMP increased the proton leak rate (measured as oligomycin-insensitive oxygen consumption rate) at all values of membrane potential;
10 it increased the proton conductance of the mitochondria about 2-fold across the whole range of driving forces (Figure 2a). AMP had no effect on the overall kinetics of the reactions of substrate oxidation (not shown). AMP also doubled the proton conductance of skeletal muscle mitochondria isolated from the frog *Rana temporaria* (Figure 2b). Since the frog is an ectotherm, this indicates that the primary
15 physiological function of AMP activation is not thermogenesis.

Other nucleotide effects on mitochondrial proton conductance have been reported. The classic example is purine nucleotide inhibition of uncoupling protein 1 (UCP1) from brown adipose tissue (Rafael, J., Ludolph, H.-J., & Hohorst, H.-J. *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 1121-1131 (1969)). UCP1 has high affinity
20 for GDP, ATP and ADP, and there is also inhibition by AMP, with $K_{0.5}$ of 110 μ M (Huang, S.-G. & Klingenberg, M. *Biochemistry*, **34**, 349-360 (1995)). The AMP effect on UCP1 appears to reflect the relatively broad specificity of inhibition by purine nucleotides, unlike the AMP activation in skeletal muscle, which is highly AMP
25 specific. The UCP1 homologues UCP2 and UCP3 have been proposed to act as uncouplers (Bouillaud, F. *Int. J. Obesity* **23**, Suppl. 6, S19-S23 (1999)) (although this is controversial (Brand, M.D., Brindle, K.M., Buckingham, J.A., Harper, J.A., Rolfe, D.F.S. & Stuart, J.A. *Int. J. Obesity* **23**, Suppl. 6, S3-S11 (1999), Cadenas, S., Buckingham, J.A., Samec, S., Seydoux, J., Dulloo, A.G. & Brand, M.D. *Int. J.*
30 *Obesity* **23**, suppl. 5, p. S99 (1999)) and there is some evidence that they are regulated by nucleotide binding (Nègre-Salvayre, A., Hirtz, C., Carrera, G., Cazenave, R., Trolly, M., Salvayre, R., Pénicaud, L. & Casteilla, L. *FASEB J.* **11**, 809-815 (1997), Echtay, K.S., Liu, Q., Caskey, T., Winkler, E., Frischmuth, K., Bienengraber, M. & Klingenberg, M. *FEBS Lett.* **450**, 8-12 (1999)). CMP stimulates
35 mitochondrial proton conductance, but at higher concentrations than tested here: $K_{0.5}$ was about 4 mM (Jekabsons, M. & Horwitz, B.A. *FASEB J.* **12**, A813 (1998)).

Purine nucleotides and carboxyatractylate inhibit the high proton conductance of foetal liver mitochondria (Valcarce, C. & Cuezva, J.M. *FEBS Lett.* **294**, 225-228 (1991)). ATP stimulates a proton conductance pathway in yeast mitochondria, but AMP has no effect at the concentrations used here (Prieto, S., Bouillaud, F. & Rial, E. *Arch. Biochem. Biophys.* **334**, 43-49 (1996)).

The possibility that the AMP activation of proton conductance was mediated by homologues of UCP1 was investigated. It has recently been shown that 24-h starvation of rats led to increased amounts of UCP2 and UCP3 mRNA in skeletal muscle, and UCP3 protein in skeletal muscle mitochondria, but did not alter proton conductance (Cadenas, S., Buckingham, J.A., Samec, S., Seydoux, J., Dulloo, A.G. & Brand, M.D. *Int. J. Obesity* **23**, suppl. 5, p. S99 (1999)). Figure 2c shows that the stimulatory effect of 1 mM AMP was not increased despite the twofold increase in UCP3 protein measured by Western blot (Cadenas, S., Buckingham, J.A., Samec, S., Seydoux, J., Dulloo, A.G. & Brand, M.D. *Int. J. Obesity* **23**, suppl. 5, p. S99 (1999)) in the same mitochondrial preparations. This observation suggests that AMP does not activate proton conductance by effects on UCP3.

The possibility that the AMP activation of proton conductance was mediated by another member of the mitochondrial inner membrane transporter family, the adenine nucleotide carrier, which exchanges cytosolic ADP for mitochondrial ATP across the mitochondrial inner membrane (Klingenberg, M. *Enzymes Biol. Membr.* **4**, 511-553 (1985)) was investigated. This carrier has a single binding site which alternately faces the cytosol or the matrix. The binding site is described as specific for the substrates, ADP and ATP (Klingenberg, M. *Enzymes Biol. Membr.* **4**, 511-553 (1985)) but it may also bind (Huber, T., Klingenberg, M. & Beyer, K. *Biochemistry* **38**, 762-769 (1999)) or transport (Kiviluoma, K.T., Peuhkurinen, K.J. & Hassinen, I.E. *Biochim. Biophys. Acta*, **974**, 274-281 (1989)) AMP. The diagnostic feature of the carrier is its specific inhibition by atractylate, carboxyatractylate and bongkrekate. It was found that the increase in muscle mitochondrial state 4 respiration rate caused by 1 mM AMP was prevented and reversed by 22.5 nmol atractylate or 2.4 nmol carboxyatractylate per mg mitochondrial protein, strongly implicating the adenine nucleotide carrier in the effect of AMP. Stimulation by 200 μ M AMP was abolished by 1 mM ATP or 1 mM ADP but not by the other purine or pyrimidine nucleotides listed above (which are not good substrates for the adenine nucleotide carrier), supporting this conclusion. There was no time-dependence of

the activation or of the carboxyatractylate inhibition, suggesting that AMP activation was directly on the adenine nucleotide carrier and not a result of AMP uptake followed by activation of a different target within the mitochondria. Fatty acids activate proton leak through the adenine nucleotide carrier (Skulachev, V.P. *Biochim. Biophys. Acta* **1363**, 100-124 (1998)), but two lines of evidence suggest that the AMP activation is different: AMP activation occurred in the presence of albumin (which chelates fatty acids) and unlike fatty acid activation, was much less in liver mitochondria (Figure 2d).

10 An interesting correlation emerged from the tissue distribution of the AMP activation of proton conductance by the adenine nucleotide carrier. Unlike skeletal muscle, there was little or no effect of 1 mM AMP on proton conductance in rat liver mitochondria (Figure 2d). The stimulation of state 4 respiration by 1 mM AMP in mitochondria from four rat tissues was 55% in skeletal muscle, 41% in heart, 27% in kidney and 7% in liver. Two isoforms of the adenine nucleotide carrier (ANC1 and ANC3) with 98% amino acid sequence homology have been identified in rats. ANC1 as a proportion of total ANC mRNA is 81% in skeletal muscle, 63% in heart, 35% in kidney and 25% in liver (Dörner, A., Olesch, M., Giessen, M., Pauschinger, M. & Schultheiss, H.-P. *Biochim. Biophys. Acta* **1417**, 16-24 (1999)). The identical ranking of AMP potency and relative ANC1 mRNA abundance in these four tissues raises the hypothesis that it is ANC1 that is involved in the AMP stimulation of mitochondrial proton conductance.

25 The AMP-activated proton conductance of the adenine nucleotide carrier is high. From Figure 2 it is about 300 nmol H⁺/min/mg protein in state 4; about 35% of the ADP/ATP turnover rate of the carrier in state 3. In brown adipose tissue mitochondria, UCP1 increases basal proton conductance up to 25-fold (Nicholls, D.G. *Eur. J. Biochem.* **77**, 349-356 (1977)). Basal proton conductance is quantitatively similar in mitochondria from rat skeletal muscle and guinea pig brown adipose (compare Figure 2a with Figure 3 in [Nicholls, D.G. *Eur. J. Biochem.* **77**, 349-356 (1977)]) and the content of UCP1 in cold-adapted brown adipose tissue mitochondria (0.5-0.8 nmol/mg protein) (Nicholls, D.G. *Eur. J. Biochem.* **77**, 349-356 (1977)) is similar to the content of adenine nucleotide carrier in skeletal muscle mitochondria (1.5 nmol/mg protein (Letellier, T., Malgat, M. & Mazat, J.-P. *Biochim. Biophys. Acta* **1141**, 58-64 (1993)), so proton transport through the AMP-activated

pathway on the adenine nucleotide carrier is about 3-4% of the maximum rate of proton transport by UCP1.

From the study of AMP analogues referred to previously, three compounds (5 available from Sigma Chemical Company Ltd, Fancy Road, Poole Dorset, England) were identified which demonstrated significant activity in decreasing membrane potential. The magnitude of the effect on membrane potential compared to the effect of AMP under the same conditions (AMP defined as 100%) was determined. The potency of the compounds on membrane potential was also determined and 10 expressed as an IC₅₀ value (AMP determined to be 93 micromoles). The details are given below:

	1) 6-Chloropurineriboside 5'-monophosphate	93.02%	170 micromol
15	2) Cordecypin 5'-monophosphate	58.98%	163 micromol
	3) Xanthosine 5'-monophosphate	61.74%	119 micromol.

These results demonstrate that the assays of the present invention are 20 suitable for identifying compounds with the desired activity. Compounds which increase proton leak are suitable as potential treatments for obesity and related comorbid conditions.

Since proton leak in muscle is a significant contributor to standard metabolic 25 rate (Rolfe, D.F.S., Newman, J.M.B., Buckingham, J.A., Clark, M.G. & Brand, M.D. *Am. J. Physiol.* 276, C692-C699 (1999)), AMP stimulated proton conductance in muscle might form part of a physiological mechanism of acute regulation of energy dissipation and standard metabolic rate, potentially changing it by 5-10%. Sustained changes in metabolic rate of this magnitude could have dramatic effects on body 30 weight, so the AMP-stimulated proton conductance of skeletal muscle mitochondria is a potential target for anti-obesity and anti-cachexia pharmaceuticals.

Figure 1 a. Effect of AMP on resting respiration of rat skeletal muscle mitochondria. Assays in a Clark oxygen electrode contained 0.5 mg mitochondrial protein/ml, 120 35 mM KCl, 5 mM KH₂PO₄, 3 mM HEPES, 2 mM MgCl₂, 1 mM EGTA, 0.3% defatted BSA, 5 µM rotenone, 1 µg/ml oligomycin and 4 mM succinate, pH 7.2, 37°C. Mitochondria had respiratory control ratios of 4.2 ± 0.4 (S.D.) Data are means ±

S.E.M. for $n = 6-12$ (except at $50 \mu\text{M}$ AMP where $n = 2$). **b.** Hanes plot of the data in **a.** $r^2 = 0.98$. The intercept on the horizontal axis gives $K_{0.5}$ for AMP = $80 \mu\text{M}$.

Figure 2 Effect of AMP on mitochondrial proton conductance. Mitochondria were
5 from **a.** skeletal muscle from rats fed *ad lib*; **b.** skeletal muscle from frog (*Rana temporaria*); **c.** skeletal muscle from 24 h-starved rats; **d.** rat liver. 1 mM AMP was absent (closed circles) or present (open circles). Respiration rate and membrane potential were measured simultaneously using electrodes sensitive to oxygen and to the potential-dependent probe TPMP*. For **a** and **c**, rat skeletal muscle mitochondria
10 were incubated as in Figure 1 with 80 ng/ml nigericin and $50 \mu\text{M}$ diadenosine pentaphosphate. The TPMP electrode was calibrated with sequential additions up to $2 \mu\text{M}$ TPMP. Malonate was sequentially added up to 2 mM to change mitochondrial potential. After each run, $0.2 \mu\text{M}$ FCCP was added to release TPMP for baseline correction. TPMP binding correction was $0.35 (\mu\text{l/mg protein})^{-1}$. Data are mean \pm
15 S.E.M. of **a**, seven or **c**, three experiments performed in triplicate. For **b**, frog mitochondria (preparation based on ref. 25) were incubated as in **a** but at 1 mg protein/ml , 5 mM succinate, without diadenosine pentaphosphate, pH 7.4, 25°C . TPMP was added up to $2.55 \mu\text{M}$, and malonate up to 5.3 mM . $2.9 \mu\text{M}$ FCCP was used and TPMP binding was assumed to be $0.4 (\mu\text{l/mg protein})^{-1}$. Data are mean
20 and range of two experiments performed in duplicate. For **d**, liver mitochondria were incubated as in **a** but at 1 mg protein/ml . TPMP was added up to $5 \mu\text{M}$, and malonate up to 5 mM . $2 \mu\text{M}$ FCCP was used and TPMP binding was assumed to be $0.4 (\mu\text{l/mg protein})^{-1}$. Data are mean and range of two experiments performed in triplicate.

Claims

1. A regulatory site for a mitochondrial proton leak wherein the site is activated by adenosine monophosphate (AMP).
5
2. The use of a regulatory site according to claim 1 in a screening assay to identify compounds which are useful in the treatment of a body weight disorder such as obesity or cachexia or related co-morbid conditions.
- 10 3. A screening method for the identification of compounds which modulate an AMP-sensitive regulatory site on mitochondria comprising the steps of:
 - a) contacting a test compound with mitochondria in the presence of a substrate for respiration in the presence of a buffer system;
 - b) measuring an index of metabolic rate; and
 - 15 c) identifying compounds which modulate the metabolic rate.
4. A method according to claim 3 which further comprises the steps of
 - a) contacting the compounds identified in claim 3 with mitochondria in the presence of a substrate for respiration in the presence of a buffer system and
20 in the presence of AMP and measuring an index of metabolic rate; and
 - b) comparing the metabolic rate in claim 3 step (b) and claim 4 step (a) and identifying compounds where there is not an additive effect on metabolic rate as compounds which modulate the AMP-sensitive regulatory site.
- 25 5. A method according to claim 3 or 4 wherein in step c) compounds which increase the metabolic rate are identified as compounds which activate an AMP-sensitive regulatory site on mitochondria.
- 30 6. A method according to claim 3 or 4 wherein in step c) compounds which decrease the metabolic rate are identified as compounds which inhibit an AMP-sensitive regulatory site on mitochondria.
7. A screening method for the identification of compounds which modulate an AMP-sensitive regulatory site on mitochondria comprising the steps of:
 - 35 a) contacting a test compound with mitochondria in the presence of a substrate for respiration in the presence of a buffer system;

- b) measuring the membrane potential; and
 - c) identifying compounds which change the membrane potential.
8. A method according to claim 7 which further comprises the steps of:
- 5 a) contacting the compounds identified in claim 7 with mitochondria in the presence of a substrate for respiration in the presence of a buffer system and in the presence of AMP and measuring membrane potential, and
- b) comparing the membrane potential in claim 7 step (b) and claim 8 step (a) and identifying compounds where there is not an additive effect on
- 10 membrane potential as compounds which modulate the AMP-sensitive regulatory site.
9. A method according to claim 7 or 8 wherein in step c) compounds which decrease the membrane potential are identified as compounds which activate
- 15 an AMP-sensitive regulatory site on mitochondria.
10. A method according to claim 7 or 8 wherein in step c) compounds which increase the membrane potential are identified as compounds which inhibit
- 20 an AMP-sensitive regulatory site on mitochondria.
11. A screening method for the identification of compounds which modulate an AMP-sensitive regulatory site on mitochondria comprising the steps of:
- a) contacting a test compound with mitochondria in the presence of a substrate for respiration in the presence of a buffer system;
- 25 b) measuring an index of metabolic rate and measuring the membrane potential; and
- c) identifying compounds which change the metabolic rate and change the membrane potential as compounds which modulate the AMP-sensitive regulatory site.
- 30
12. A method according to claim 11 which further comprises the steps of
- a) contacting the compounds identified in claim 11 with mitochondria in the presence of a substrate for respiration in the presence of a buffer system and in the presence of AMP, measuring an index of metabolic rate and measuring
- 35 the membrane potential; and

- b) comparing metabolic rate and the membrane potential in claim 11 step (b) and claim 12 step (a) and identifying compounds where there is not an additive effect on metabolic rate and membrane potential as compounds which modulate the AMP-sensitive regulatory site.
- 5
13. A method according to claim 11 or 12 wherein in step c) compounds which increase the metabolic rate and decrease the membrane potential are identified as compounds which activate an AMP-sensitive regulatory site on mitochondria.
- 10
14. A method according to claim 11 or 12 wherein in step c) compounds which decrease the metabolic rate and increase the membrane potential are identified as compounds which inhibit an AMP-sensitive regulatory site on mitochondria.
- 15
15. A method according to any one of claims 3 to 6 and 11 to 14 wherein the index of metabolic rate is oxygen consumption.
- 20
16. A method according to any one of claims 3 to 15 wherein the mitochondria are isolated mitochondria or a suitable part or derivative thereof.
17. A method according to any one of claims 3 to 15 wherein the mitochondria are skeletal muscle mitochondria or a suitable part or derivative thereof.
- 25
18. A method according to claim 17 wherein the skeletal muscle mitochondria are rat skeletal muscle mitochondria.
19. A method according to any one of claims 3 to 15 wherein the mitochondria are present in intact eukaryotic cells.
- 30
20. A method according to claim 19 wherein the intact cells present in tissue slices of mammalian origin or cell lines of mammalian origin.
- 35
21. A method according to any one of claims 3 to 15 wherein a complex 1 inhibitor is present.

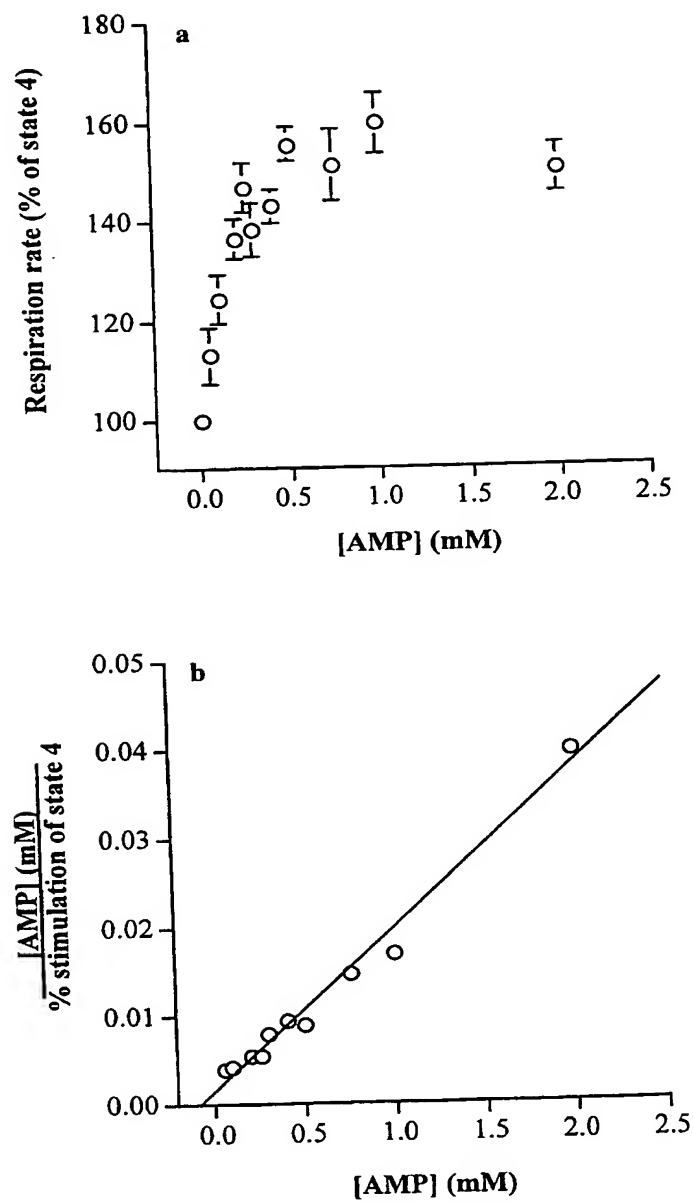
22. A method according to any one of claims 3 to 15 wherein the substrate is a succinate salt.
23. A method according to claim 21 wherein complex 1 inhibitor is rotenone.
- 5 24. A method according to any one of claims 3 to 22 wherein the screening method is carried out in the presence of varying concentrations of an electron transport inhibitor.
- 10 25. A method according to claim 24 wherein the electron transport inhibitor is selected from a malonate salt, myxothiazol or a cyanide salt.
26. A method according to claim 24 wherein the electron transport inhibitor is a malonate salt.
- 15 27. A screening method according to claim 15 wherein the oxygen consumption is measured by an oxygen electrode.
28. A screening method according to any one of claims 9 to 26 wherein the membrane potential is measured using ion selective electrodes.
- 20 29. A screening method according to any one of claims 9 to 26 wherein the membrane potential is measured using fluorescent membrane potential dyes.
- 25 30. A screening method according to any previous claim wherein an inhibitor of ATP synthesis is present.
31. A method according to any one of claims 3 to 30 for the identification of compounds which are suitable for use in the treatment of a body weight disorder.
- 30 32. A method according to any one of claims 5, 9, and 13 for the identification of compounds which are suitable for use in the treatment of obesity and related conditions.

33. A method according to any one of claims 6, 10 and 14 for the identification of compounds which are suitable for use in the treatment of cachexia and related conditions.
- 5 34. A screening method for the identification of compounds which are useful in the treatment of a body weight disorder the method comprising the step of identifying a compound which selectively binds to ANC.
- 10 35. A screening method for the identification of compounds which are useful in the treatment of obesity and related conditions the method comprising the step of identifying an agonist of ANC.
- 15 36. A screening method for the identification of compounds which are useful in the treatment of cachexia and related conditions the method comprising the step of identifying an antagonist of ANC.
37. A binding assay for the identification of compounds which are suitable for use in the treatment of obesity and related conditions comprising the steps of:
- 20 a) incubating an ANC-containing preparation with a labelled ligand to produce a labelled ANC-containing preparation;
- b) contacting a test compound with the labelled ANC-containing preparation; and
- c) identifying a compound which reduces the amount of labelled ligand present in the ANC-containing preparation as a compound which may suitable for use in the treatment of obesity.
- 25 38. An assay according to claim 37 in which the ANC-containing preparation comprises one of the following: a) intact tissue preparations b) cell lines, from a skeletal muscle source or from a cardiac source or from an aortic smooth muscle source c) cells into which ANC has been introduced by genetic means d) cells isolated from tissues for example from cardiac or skeletal muscle tissues e) membranes ; f) mitochondria ; g) mitochondrial membranes or h) isolated ANC preferably in purified form.
- 30

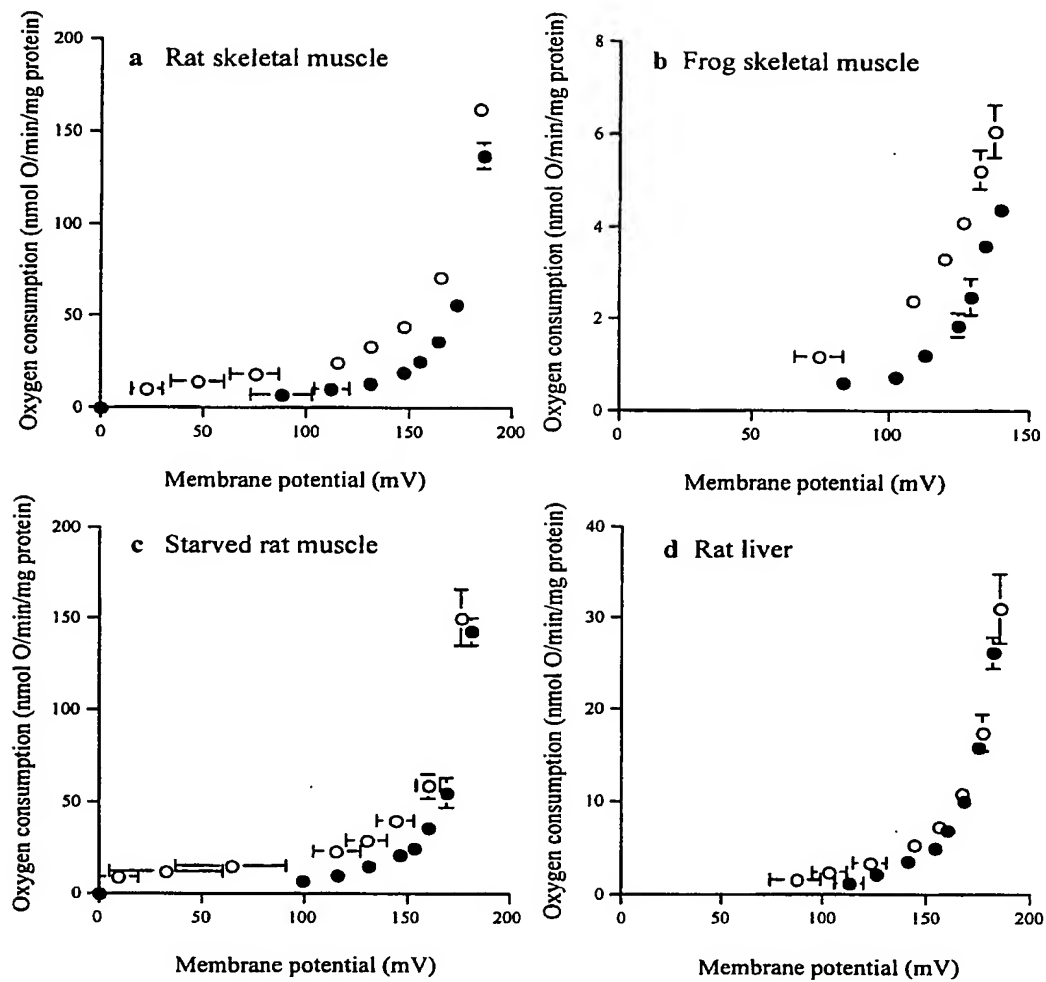
39. An assay according to either claim 37 or claim 38 in which the ANC-containing preparation is a cell line which is optionally upregulated.
40. An assay method according to any one of claims 37 to 39 in which the
5 labelled ligand is radiolabelled or fluorescently labelled atractylate or fluorescently labelled ATP or ADP.
41. A screening method for the identification of compounds which are useful in
10 the treatment of a body weight disorder the method comprising the step of identifying a compound which modulates the mitochondrial proton leak mediated by an ANC.
42. A screening method for identification of compounds which are useful in the
15 treatment of obesity and related conditions the method comprising the step of identifying a compound which enhances mitochondrial proton leak mediated by an ANC.
43. A screening method for identification of compounds which are useful in the
20 treatment of cachexia and related conditions the method comprising the step of identifying a compound which reduces mitochondrial proton leak mediated by an ANC.
44. A (functional) screening method for identifying compounds which modulate
the proton leak mediated by an ANC comprising the steps of :
- 25 a) incubating a test compound with cells in which an ANC is upregulated and measuring an index of metabolic rate or membrane potential
- b) incubating a test compound with control cells in which the ANC used in step
a) is absent or is present at lower levels than in step a) and measuring an
index of metabolic rate or membrane potential and
- 30 c) identifying a compound which gives rise to a different metabolic rate or
different membrane potential in step a) compared to step b) as a compound
which modulates the proton leak mediated by an ANC.
45. A method according to claim 44 wherein in step c) a compound which gives
35 rise to an increased metabolic rate or decreased membrane potential is
identified as a compound which enhances the proton leak mediated by an
ANC.

46. A method according to claim 44 wherein in step c) a compound which gives rise to a decreased metabolic rate or increased membrane potential is identified as a compound which reduces proton leak mediated by an ANC.
- 5 47. A method according to any one of claims 44 to 46 for the identification of compounds which are suitable for use in the treatment of a body weight disorder.
- 10 48. A method according to claim 45 for the identification of compounds which are suitable for use in the treatment of obesity and related conditions.
49. A method according to claim 46 for the identification of compounds which are suitable for use in the treatment of cachexia and related conditions.
- 15 50. A method or assay according to any one of claims 3 to 49 further comprising the step of screening a compound identified as being suitable for use in the treatment of a body weight disorder in a further screen for suitability in treating a body weight disorder.
- 20 51. A method or assay according to any one of claims 5, 9, 13, 35, 42, 45 and 48 further comprising the step of screening a compound identified in a further screen for suitability in treating obesity or a related condition.
- 25 52. A method or assay according to any one of claims 6, 10, 14, 36, 43, 46 and 49 further comprising the step of screening a compound identified in a further screen for suitability in treating cachexia or a related condition.
- 30 53. A compound identifiable in a screening method or assay according to any one of claims 3 to 52.
54. A compound identified in a screening method or assay according to any one of claims 3 to 52.
- 35 55. A compound according to claim 53 or 54 for use in medicine.

56. A method for treating a body weight disorder in a patient the method comprising administering to the patient a compound according to claim 53 or 54.
- 5 57. Use of a compound according to claim 53 or 54 in the manufacture of a medicament for treating a body weight disorder.
58. A method of treating a patient with obesity or a related co-morbid condition the method comprising administering to the patient an agonist of an AMP-sensitive regulatory site on mitochondria or an agonist of the AMP effect on the ANC mediated proton leak.
- 10
59. Use of an agonist of an AMP-sensitive regulatory site on mitochondria or an agonist of the AMP effect on the ANC mediated proton leak in the manufacture of a medicament for treating obesity or a related co-morbid condition.
- 15
60. A method according to claim 58 or the use according to claim 59 wherein the activator of an AMP-sensitive regulatory site on mitochondria is any one of 6-chloropurineriboside 5'-monophosphate, cordecypin 5'-monophosphate and xanthosine 5'-monophosphate.
- 20

**FIGURE 1**

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**FIGURE 2**

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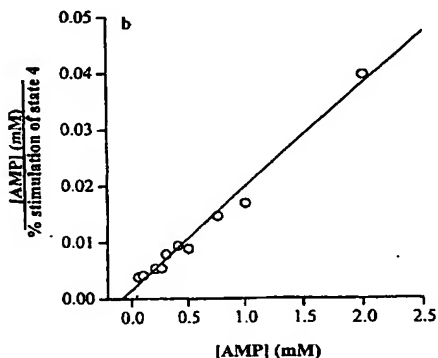
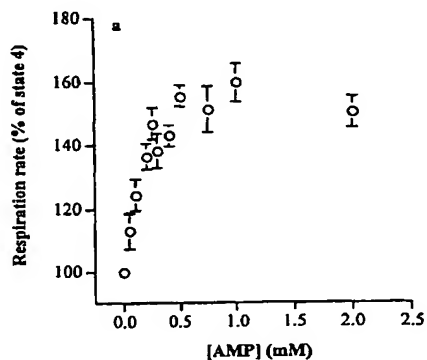
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[Continued on next page]

(54) Title: **SCREENING FOR COMPOUNDS MODULATING MITOCHONDRIAL PROTON LEAK**



(57) Abstract: The present invention provides a screening method for the identification of compounds which modulate an AMP-sensitive regulatory site on mitochondria comprising the steps of: a) contacting a test compound with mitochondria in the presence of a substrate for respiration in the presence of a buffer system; b) measuring an index of metabolic rate; and c) identifying compounds which modulate metabolic rate. Alternatively or additionally membrane potential can be measured. The invention also comprises a regulatory site for a mitochondrial proton leak which is related to the adenine nucleotide carrier, a binding assay and a functional assay.



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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/05863

A. CLASSIFICATION OF SUBJECT MATTER

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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, WPI Data, PAJ, EMBASE, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JEKABSONS M ET AL: "Effect of nucleotides on rat liver and skeletal muscle mitochondria non-phosphorylating respiration and membrane potential." FASEB JOURNAL, vol. 12, no. 5, 20 March 1998 (1998-03-20), page A813 XP002154017 Annual Meeting of the Professional Research Scientists on Experimental Biology 98, Part II; San Francisco, California, USA; April 18-22, 1998 ISSN: 0892-6638 cited in the application	1,3,5-7, 11, 15-23, 27,29-33
A	left-hand column, paragraph 4714	1-3, 34-36, 41-43, 50-52
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☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

Inter- national Application No
PCT/EP 00/05863

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>MONEMDJOU S ET AL: "Mitochondrial proton leak in brown adipose tissue, mitochondria of UCP1-deficient mice is GDP insensitive" AMERICAN JOURNAL OF PHYSIOLOGY: ENDOCRINOLOGY AND METABOLISM, June 1999 (1999-06), pages E1073-E1082, XP002154018 abstract page E1074, left-hand column, line 7 -page E1075, left-hand column, line 42 page E1075; figure 1 page E1076; figure 3</p>	<p>1,3,6,7, 10,11, 14-23, 25-28, 30,31,33</p>
A		<p>1,3,36, 41,43, 50,52</p>
A	<p>----- EARNSHAW M J ET AL: "THE ADENINE NUCLEOTIDE CARRIER OF PLANT MITOCHONDRIA CONTRACTION INDUCED BY ADP" PLANT SCIENCE LETTERS, vol. 6, no. 5, 1976, pages 343-348, XP000972265 ISSN: 0304-4211 the whole document</p>	<p>1-4,21, 34</p>
A	<p>----- BOUILLAUD F ET AL: "A sequence related to a DNA recognition element is essential for the inhibition by nucleotides of proton transport through the mitochondrial uncoupling protein." EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL, vol. 13, no. 8, 1994, pages 1990-1997, XP002154020 ISSN: 0261-4189 abstract page 1994; figure 7</p>	<p>1-4,7,9, 11,13, 29,32, 34,35, 41,42, 50,51</p>
X	<p>----- BLOCK M R ET AL: "INTERACTION OF 3'-O-1 NAPHTHOYL ADP A FLUORESCENT ADP ANALOG WITH THE ADP ATP CARRIER PROTEIN IN THE MITOCHONDRIAL MEMBRANE" BIOCHEMISTRY, vol. 21, no. 22, 1982, pages 5451-5457, XP002154021 ISSN: 0006-2960 abstract</p> <p>----- -/--</p>	<p>37-40</p>

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/05863

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	CADENAS S ET AL: "AMP decreases the efficiency of skeletal-muscle mitochondria" BIOCHEMICAL JOURNAL, vol. 351, no. 2, 15 October 2000 (2000-10-15), pages 307-311, XP000974944 the whole document	1-52
X	----- PARES, XAVIER ET AL: "The reaction of bovine pancreatic ribonuclease A with 6-chloropurineriboside 5'-monophosphate. Evidence on the existence of a phosphate-binding sub-site" EUR. J. BIOCHEM., vol. 105, no. 3, 1980, pages 571-580, XP000974761	53-55
A	abstract -----	57, 59

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 56,58 and 60 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound.

Continuation of Box I.1

Claims Nos.: 56,58,60

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

Continuation of Box I.2

Present claims 53-55 relate to compounds defined by reference to a desirable characteristic or property, namely that these compounds are identifiable in a screening method according to any one of claims 3-52. Claim 56 relates to a method using these compounds and claim 57 relates to a use of these compounds.

The claims cover all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compounds by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the compounds mentioned in the description at pages 28. lines 4-22.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

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